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The specific aim o f this work is to develop camptothecin (CPT) analogs for treatment of women with breast cancer. We evaluated these analogs using three biochemical tests: (i) inhibition of growth of breast cancer cells, (ii) induction of topoisomerase I-mediated cleavage of DNA, and (iii) reversal of DNA cleavage. Our results indicate that the 10,11-methylenedioxy CPT analog was effective in all three tests. Further, this analog was effective against MX-1 and MDA-231 human breast xenografts growing in nude mice. The 10,11-methylenedioxy analog appears to be the best choice among the analogs examined, and we are currently pursuing moving this compound into clinical trials.

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A. INTRODUCTION TO PART I: Biochemical and Biological Evaluation of Novel Camptothecin Analogs with Activity Against Breast Cancer.

A.1 Background.

In 1966, Wall and colleagues discovered that camptothecin (CPT; Fig. 1) was the component in the extract from the stem of the Chinese tree *Camptotheca acuminata* [1] active against L1210 murine leukemia cells. Early clinical trials with CPT in the late 1960s showed that this plant alkaloid had activity against a variety of solid tumors [2-4]. However, further development was discontinued because of unpredictable and severe myelosuppression, gastrointestinal toxicity, and hemorrhagic cystitis.

Further development of topoisomerase I (topo I) inhibitors for cancer therapy was stimulated by the characterization of CPT as a specific topo I inhibitor [5, 6]. Topo I relaxes DNA supercoiling by making transient single-strand breaks [7, 8]. These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex [5, 6]. CPT and analogs specifically and reversibly stabilize cleavable complexes by inhibiting their religation (reviewed in [9, 10]). The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes [6, 11, 12].

There are now several topo I inhibitors at various stages of clinical development. One of these, irinotecan (CPT-11), has recently been approved by the U.S. Food and Drug Administration for the second-line treatment of metastatic colorectal cancer. CPT-11 is a pro-drug and is converted to the active SN-38 (7-ethyl-10-hydroxycamptothecin) by carboxylesterases to exert its antitumor activity [13, 14].

The potent activity of SN-38 has led to the development of a number of 7- and 10-substituted camptothecins [15-19]. These include the highly potent 10,11-methylenedioxycamptothecin (MDCPT) and 7-chloromethyl-10,11-methylenedioxycamptothecin (CMMD; Fig. 1). The latter compound is of particular interest as it is capable of forming a covalent complex with DNA through nucleophilic displacement of the chlorine moiety by DNA while in the cleavable complex [18, 20].

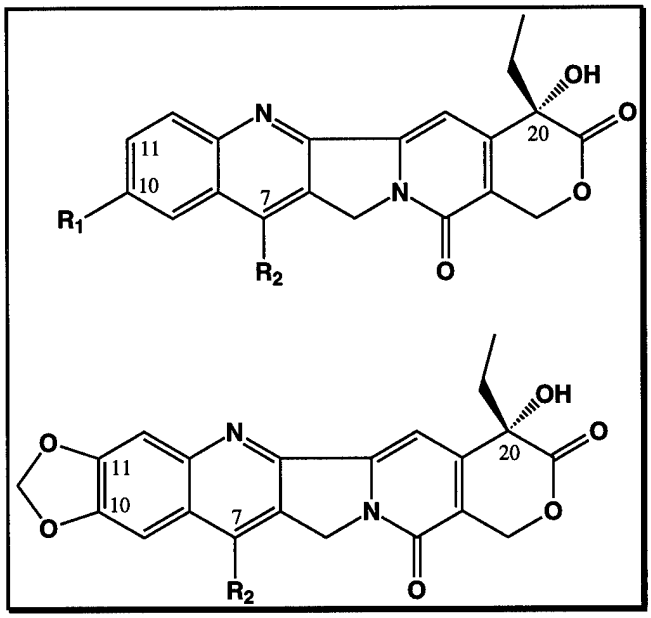

Figure 1. The molecular structure of the camptothecin analogs used in these studies. CPT (top): R_1 = H, R_2 = H. C1CPT through C4CPT: R_1=H, R_2= methyl, ethyl, propyl, or butyl. OHCPT: R_1 = OH, R_2 = H. OHC1CPT through OHC4CPT: R_1 = OH, R_2 = methyl, ethyl, propyl, or butyl. OMeCPT: R_1 = OCH_3, R_2 = H. OMeC1CPT through OMeC4CPT: R_1 = OHC_3, R_2 = methyl, ethyl, propyl, or butyl. MDCPT (bottom): R_2 = H. MDC1CPT through MDC4CPT, R_2 = methyl, ethyl, propyl, or butyl.

A.2 Hypothesis/Purpose

It has been suggested that the property of the 7- and 10-substituted analogs thought to be most relevant to their potent antitumor activity is the slow reversal of the cleavable complexes formed with these drugs [18, 21, 22]. Compared to CPT, the 10-hydroxycamptothecin (OHCPT), SN-38, MDCPT, and CMMD all appeared to have a longer-lived cleavable complex. As these compounds are all substantially more cytotoxic than CPT, it has been postulated that, since CPT toxicity is a time-dependent phenomenon, the persistence of cleavable complexes may be an essential property for induction of protein-linked DNA strand breaks and drug potency [18, 21].

In order to understand the relationship between CPT substitution, cytotoxicity, and cleavable complex reversibility, we have systematically synthesized and evaluated a series of 7-alkyl-CPT, 7-alkyl-10-hydroxy-CPT, 7-alkyl-10-methoxy-CPT, and 7-alkyl-10,11-methylenedioxy-CPT that have incremental lengths in the 7-alkyl chains. These analogs have been characterized with respect to: (i) their ability to induce topo I-mediated cleavage of plasmid DNA and DNA-protein crosslinks in CEM cell nuclei; (ii) the reversibility of the cleavable complexes formed with plasmid DNA and in CEM cell nuclei; and (iii) the growth inhibitory activity of the analogs to selected breast cancer cell lines. Our results indicate that the potency of the 7- and 10-substituted analogs does not reflect the lifetime for reversal of the cleavable complex formed with these compounds—even using analogs capable of forming covalent complexes with DNA. We demonstrate that rate constants for reversal of cleavable complexes are similar among the analogs tested. Instead, the *in vitro* biological activity of the drugs appears to more closely parallel the concentration of drug required to produce cleavable complexes in plasmid DNA under steady-state conditions.

B. MATERIALS AND METHODS FOR PART I:

B.1 Chemistry. The abbreviations used for the CPT analogs are given in Table 1 and their structures given in Fig. 1. All CPT analogs used were the synthetic 20(S)- stereoisomer. CPT, OHCPT, and OMeCPT were obtained as pure natural products [1, 23]. 7-Alkyl-CPT (C1CPT through C4CPT) were synthesized according to [24]. 7-Alkyl-OHCPT analogs (OHC1CPT through OHC4CPT) were synthesized from OHCPT by the procedure of Sawada et al [25]. The 10-methoxy analogs OMeC1CPT through OMeC4CPT were synthesized according to the procedure for the synthesis of 10-methoxycamptothecin [26]. The synthetic scheme for the preparation of MDCPT , MDC1CPT through MDC4CPT, and CMMD has also been reported previously [16, 19]. The synthesis of the compounds was documented in our two previous annual reports.

B.2 *In Vitro* Growth Inhibitory Activity. Human breast carcinoma lines MDA-231 and BT-20 were maintained in IMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 incubators until plated for use in MTT assays. Exponentially growing cells (1-2 × 10^3 cells, unless otherwise specified) in 0.1 ml medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test analogs were added in duplicate to the cell plates. After incubation at 37 °C in a humidified incubator with 5% CO_2-95% air for 3 days, the plates were centrifuged briefly and 100 µl of the growth medium was removed. Cell cultures were incubated with 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide [MTT, 1 mg/ml in Dulbecco's phosphate buffered saline (PBS)] for 4 hr at 37 °C. The resulting purple formazan precipitate was solubilized with 200 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a BioRad Model 3550 Microplate Reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance data was transferred to a PowerMacintosh and the IC_{50} values were determined by a computer program (Kaleidagraph, Synergy Software, Reading, PA) that fitted all of the data to the following four-parameter equation:

$$Y = \frac{A_{max} - A_{min}}{1 + (X/IC_{50})^n} + A_{min} \qquad \text{(eq. 1)}$$

where A_{max} is the absorbance of control cells, A_{min} is the absorbance of cells in the presence of highest agent concentration, Y is the observed absorbance, X is the agent concentration, IC_{50} is the concentration of agent that inhibits the cell growth by 50% of control cells (based on the absorbance) and n is the slope of the curve.

B.3 Cleavable Complex Formation by CPT Analogs in Plasmid DNA. CPT analog-induced cleavable complex formation was performed in 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol. A 250 ng sample of pBR322 plasmid DNA (Gibco BRL) was mixed with the drug of interest, and 4 units human topoisomerase I enzyme (Topogen, Inc.) was subsequently added to a 20 µL total mixture volume. Reaction mixtures were assembled on ice.

Each mixture was incubated at 37°C for 30 minutes, then terminated by the addition of 2 µL of 10% SDS and 2 µL of 0.5 mg/mL Proteinase K (Promega). The mixture was further incubated for another 30 minutes at 37°C, then treated with 2µL loading solution (25 % bromophenol blue, 50 % glycerol) and extracted with 20 µL CIA (Chloroform: Isoamyl Alcohol, 24:1). Following the CIA extraction, the resulting sample was analyzed by electrophoresis for 16 hours at 30 volts on a 1% agarose gel in 1% TAE buffer (pH 8.0, containing 0.5µg/ml Ethidium Bromide).

After electrophoresis, the gel was stained with 1:10,000 dilution of SYBR Green (Molecular Probes) in TE buffer (pH 8.0) and photographed under transillumination with 300 nm UV light. The resulting photograph was scanned using a Polaroid Photopad scanner and the relaxed DNA (Form I_R DNA) band quantified using NIH Image 1.6 software. A linear relationship between the amount of DNA present and the signal generated was established by quantifying increasing concentrations of supercoiled DNA.

Dose response data were fitted to a simple E_{max} model according to:

$$\text{Cleaved Complex} = \frac{E_{max}[Drug]}{EC_{50} + [Drug]} \qquad \text{(eq. 2)}$$

where $[Drug]$ is the molar concentration of analog, E_{max} is the maximal relaxed DNA signal, and EC_{50} is the concentration of drug required to produce 50% of the maximal response. Data were fitted to this equation using the nonlinear least squares routine in Kaleidagraph (Synergy Software, Reading, PA).

B.4 Reversal of Cleavable Complex Formation in Plasmid DNA. Reversal of the Topo I cleavage activity of the pBR322 plasmid DNA was accomplished by the method of [27]. Cleavable complexes were formed in the presence of sufficient analog to induce >90% nicked DNA (as determined from EC_{50} curves). The reaction protocol consisted in the preparation of one 75 µL reaction mixture constructed as described above. The reaction mixture was incubated for 30 minutes at 23°C. A 100-fold excess of sonicated salmon sperm linear DNA (Gibco BRL, 10 mg/mL) was added to the reaction mixture. Aliquots were then removed at 0.5, 1, 2, 5, 10,15, 30, 45 and 60 minute intervals. The reaction was stopped by the addition of 11 µL H_20, 2 µL 10% SDS and 2 µL Proteinase K, then incubated at 23°C for another 30 minutes. The resulting samples were further mixed with loading solution and analyzed using 1% agarose gels, as above. The percentage of cleaved DNA with respect to time was determined based on the amount of Form II (nicked) DNA present in the 0.5 sec lane.

Under these experimental conditions, the following simplified equilibrium scheme is established (the detailed equilibria are given in [27]):

$$T + S \underset{k21}{\overset{k12}{\rightleftharpoons}} TS \quad and \quad K_1 = \frac{[TS]}{[T][S]} \qquad \text{(eq. 3)}$$

and

$$T + L \underset{k31}{\overset{k13}{\rightleftharpoons}} TL \quad and \quad K_2 = \frac{[TL]}{[T][L]} \qquad \text{(eq. 4)}$$

where T is the molar concentration of topo I, S is the molar concentration of supercoiled DNA (in binding sites), and L is the molar concentration of linear DNA fragments (in binding sites). The kinetic scheme for linear DNA-mediated competition for topo I follows the general derivation for any ligand exchange reaction [28]. When the concentration of linear DNA fragments is large compared to the concentration of supercoiled DNA, the concentration of complexes *TS* will decay with a single exponential time constant, τ, that is approximately k_{12} in equation 3. All of the time-dependent decays of topo I-mediated cleavable complexes (*TS*) could be well fitted to a single exponential decay, indicating a simple pseudo first-order reaction:

$$\%\ \text{Cleaved DNA} = A\exp(-\tau t) + C \qquad \text{(eq. 5)}$$

where t is time (in min), τ is the exponential rate constant with units of min^{-1}, and A and C are constants representing amplitude and final % cleaved DNA, respectively. Eq. 3 was fit to the data with Kaleidagraph (Synergy Software, Reading, PA) to determine the rate constants τ

It is important to briefly discuss the amplitude A of the decay of cleavable complexes (*TS*) with time. At equilibrium (i.e, after an infinite time t has elapsed), the concentration of cleavable complexes remaining is given by (see [28]):

$$[TS] = \frac{1}{2K_1}\left(1 + K_2[L]_o + 2K_1[TS]_o - \sqrt{(1 + K_2[L]_o)(1 + K_2[L]_o + 4K_1[TS]_o)}\right) \qquad \text{(eq. 6)}$$

where K_1 and K_2 are given in equations 3 and 4, $[L]_o$ is the initial amount of linear DNA added, and $[TS]_o$ is the initial amount of cleavable complexes present. The amplitude of the decay ($[TS]_o$ - $[TS]$) is therefore dependent on the ratio of the binding constants K_1 and K_2. While not explicitly shown in equations 3 and 4, these binding constants are in turn dependent on the concentration of CPT analog present. The CPT analogs enhance K_1 by reducing the k_{21} value, but this reduced k_{21} depends on the concentration of analog present and the EC_{50} (~K_d) value for the analog being examined. Hence, following introduction of competing linear DNA, the cleavable complexes will decay from the initial steady state to a new steady state. We use a fixed amount of topo I, plasmid DNA, and linear DNA in the experiments. However, due to the differences in EC_{50} values for poisoning of topo I (see below), the experiments required the use of differing concentrations of CPT analogs to achieve >90% cleavable complex formation. Thus, the amplitudes of the decay are the differences in the steady-state concentrations of complexes (*TS*) in the presence of varying concentrations of CPT analogs, and therefore the amplitudes of the decay do not necessarily reflect the rate constants τ for the decay. In general, we would expect that those analogs with lower EC_{50} values would have lower amplitudes of decay, due to the CPT analog's high-affinity for the topo I-DNA binary complex. We report here only the values of τ, since these, not the amplitudes, reflect the true stability (or lifetime) of the cleavable complex.

B.5 DNA-protein crosslinks (DPC) in isolated nuclei. Trapping of the cleavable complexes of endogenous topoisomerase I in nuclei were assessed based on DPC induction measured by a standard K^+/dodecyl sulfate precipitation technique [29, 30]. Briefly, human leukemia CEM cells, grown in

Jokklik's minimal essential medium with 10% fetal calf serum were prelabeled with [^{14}C]thymidine as described elsewhere [31]. Cells were lysed in isotonic Nuclei Isolation Buffer (2 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM ethylenebis–(oxyethylenenitrilo)–tetraacetic acid (EGTA), pH 6.9) with 0.3 % (v/v) Triton X-100 followed by centrifugation (300 × g, 13 min) and resuspension in the isolation buffer at 0.5 × 10^6 nuclei/ml. Aliquots of nuclear suspension were treated with drugs as indicated. In the reversal experiments, samples were centrifuged(500 × g, 5 min), the nuclear pellets resuspended in a fresh Nuclei Isolation Buffer followed by the additional incubation as indicated. All the reactions were terminated by addition of equal volume of 3 % SDS, 40 mM EDTA, 0.4 mg/ml DNA, pH 8.0, preheated to 65°C. Further steps were performed exactly as described elsewhere [29, 30]. Separate aliquots of nuclear suspension were used to determine total radioactivity in each individual suspension [29, 30]. The results are expressed as percentage of total DNA that co-precipitated with proteins corrected for the background precipitation in control samples. The latter value typically amounted to 2-5% of total radioactivity. For the reversal experiments, the results are normalized for the DPC levels at the end of drug treatment.

C. RESULTS FOR PART I:

C.1 Cell growth inhibition by camptothecin analogs. The campothecin analogs were examined for their ability to inhibit growth of two breast carcinoma cell lines. The abbreviations for the synthesized compounds and the IC_{50} values for growth inhibition are given in Table 1. Against BT-20 and MDA-231 cell lines, the growth inhibition results parallel those of the EC_{50} data for cleavable complex formation (given below). All 10-substituted analogs inhibit cell growth more than CPT, as did the 7-alkyl CPT. For the CPT, OHCPT, and MDCPT series, the 7-methyl analogs were among the most potent in inhibiting cell growth, but longer alkyl chain lengths were also effective. The activity of the longer-chained compounds may be due to enhanced cellular accumulation of these more hydrophobic compounds.

Interestingly, the MDCPT analogs inhibited cell growth substantially more than the other analogs examined, including the OHCPT. The MDCPT growth inhibitory activity against two cell line was 2- to 40-fold higher than observed with CPT or the other analogs. This is also likely due to the enhanced cellular accumulation, as the MDCPT analogs are likely more hydrophobic than the OHCPT analogs. The compound MDC1CPT was the most active compound examined against the two lines, and this data is consistent with the EC_{50} data for topo I poisoning on plasmids (see below).

C.2 Induction of cleavable complexes by camptothecin analogs. All compounds examined exhibited a simple hyperbolic dose response in their ability to induce cleavable complexes (Fig. 2A), and this response that could be fitted well by a simple E_{max} model (equation 2). The EC_{50} values obtained from the curve fits, such as that shown for OHCPT in Fig. 2A, are recorded in Table 1 for each compound tested.

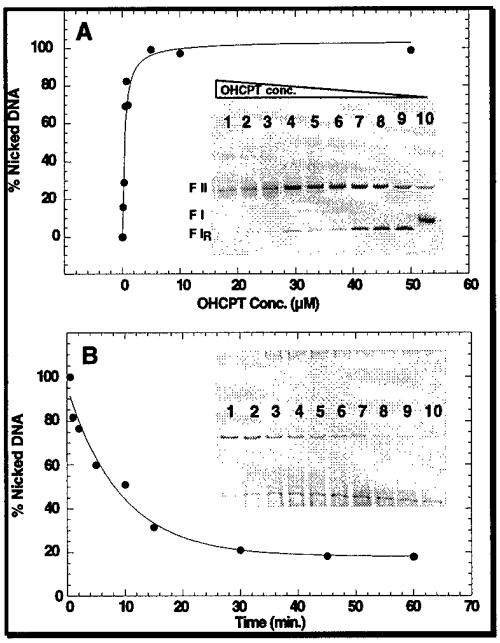

Figure 2. Induction of cleavable complexes and their reversal by 10-hydroxycamptothecin. (A) Concentration dependence of cleavable complex formation. Human topo I and pBR322 supercoiled plasmid DNA were incubated with 10-hydroxycamptothecin at 50, 10, 5, 1, 0.7, 0.5, 0.3, 0.1, and 0 μM concentrations (lanes 1-9, respectively). Lane 10 contained no drug or topo I. The upper (nicked) DNA band was quantified and expressed as percentage of total DNA. The solid curve is to the EC_{50} equation (eq. 2 in the text). The EC_{50} values determined in this manner for all analogs are recorded in Table 1. (B) Time course for reversal of cleavable complexes formed with 10-hydroxycamptothecin (OHCPT). Complexes were formed with 20 μM OHCPT, and then 100-fold linear salmon sperm DNA was added. Aliquots were withdrawn prior to DNA addition (lane 1) and 0.5, 1, 2, 5, 10, 15, 30, 45, and 60 min following DNA addition (lanes 2-10, respectively). Nicked DNA was quantified and plotted vs. time, with the solid line being the fit to eq.5 in the text. The rate constants, τ, were determined for all analogs by this method, and are given in Table 2.

In agreement with previous reports [16-19, 21], substituting the 10-position of camptothecin with a hydroxy or methoxy group, or by making the 10,11-methylenedioxy analog yielded compounds with significantly higher potency in inducing cleavable complex formation (Table 1), as compared to CPT. Substitution at the 7-position of CPT (7-alkyl CPT) also yielded compounds with more potency than CPT in inducing cleavable complex formation.

For the 7,10-disubstituted compounds, the 7-alkyl-10-methoxy compounds were only slightly more (~2-fold) potent than the 7-alkyl-CPTs in this function. Both the 10-hydroxy CPT and 10,11-methylenedioxy CPT were substantially more potent than CPT or OMeCPT as topo I poisons. Likewise, the 7-alkyl-10-hydroxy CPT and 7-alkyl-10-methylenedioxy CPT series were all highly potent compounds.

Interestingly, in the 7-alkyl CPT, OHCPT, and MDCPT series, the potency of the analogs were maximized by the presence of a 7-methyl group. Longer alkyl chains at this position were either as potent as or slightly less potent than the parent compound, with the exception of the 7-alkyl CPT, which were all more potent than CPT. The methyl group was not the most potent analog of the OMeCPT series, where the length of the 7-alkyl chain did not appear to affect the potency of these compounds.

Table 1. Potency of camptothecin analogs in inhibiting growth of breast cancer tumor cell lines and in poisoning topoisomerase I.

Camptothecin Analog	IC_{50} growth inhibition vs. BT-20 cells (nM)	IC_{50} growth inhbition vs. MDA-231 cells (nM)	EC_{50} for topo I poisoning (μM)
CPT	>500	>500	18.85 ± 12.82
C1CPT	33.1 ± 11.9	1.03 ± 0.71	1.07 ± 0.34
C2CPT	61.3 ± 21.3	2.22 ± 1.98	4.44 ± 1.86
C3CPT	120.2 ± 50.3	4.74 ± 1.34	7.19 ± 1.01
C4CPT	24.8 ± 8.9	1.39 ± 0.75	7.22 ± 2.85
OHCPT	34.3 ± 9.8	7.27 ± 4.37	0.35 ± 0.07
OHC1CPT	18.2 ± 5.4	2.13 ± 2.05	0.13 ± 0.02
OHC2CPT	50.1 ± 14.0	4.32 ± 1.97	0.32 ± 0.04
OHC3CPT	35.2 ± 34.2	4.25 ± 1.23	0.60 ± 0.16
OHC4CPT	8.8 ± 1.5	0.73 ± 0.38	0.53 ± 0.06
OMeCPT	43.5 ± 3.8	4.12 ± 1.76	0.72± 0.26
OMeC1CPT	17.5 ± 6.0	1.67 ± 1.24	2.40 ± 0.45
OMeC2CPT	19.0 ± 1.7	2.32 ± 1.55	2.82 ± 0.74
OMeC3CPT	11.3 ± 2.1	3.07 ± 0.61	4.51 ± 1.59
OMeC4CPT	ND	5.33 ± 5.78	2.85 ± 0.94
MDCPT	7.5 ± 2.5	1.19 ± 0.29	0.29 ± 0.14
C1MDCPT	1.2 ± 0.5	0.02 ± 0.01	0.03 ± 0.02
C2MDCPT	4.4 ± 1.0	0.71 ± 0.17	0.66 ± 0.50
C3MDCPT	2.2 ± 0.5	0.92 ± 0.44	0.45 ± 0.07
C4MDCPT	2.3 ± 1.0	0.68 ± 0.53	0.51 ± 0.10
CMMD (chloromethyl)	19.3 ± 1.7	0.54 ± 0.33	0.31 ± 0.05

C.3 Induction of Protein-DNA crosslinks in CEM cell nuclei. To extend the findings with purified enzyme and plasmid DNA, we examined the effects of selected CPT analogs (CPT, MDCPT, MDC2CPT, and CMMD) in isolated human leukemic CEM cell nuclei. Drug-induced trapping of the cleavable complexes in nuclei was assessed based on induction of DNA-protein crosslinks (DPC). This

system allows one to monitor drug effects on endogenous topoisomerase I on its natural target--nuclear chromatin [30].

The results indicate that all the examined analogs are more potent inducers of DPC than CPT. For example, 1 μM CPT was needed to produce a significant DPC level (35.3±11 % total DNA) after a 30 min incubation of nuclei with drug. In contrast, similar or higher DPC levels were induced by the other drugs at 0.1 μM (53.6±8.2, 36.3, and 55.8±1.9 % total DNA for MDC2CPT, MDCPT, and CMMD, respectively, Fig. 3). Also, whereas DPC induced by CPT plateau after a few minutes incubation, DPC by CMMD continue to increase until 30 min.

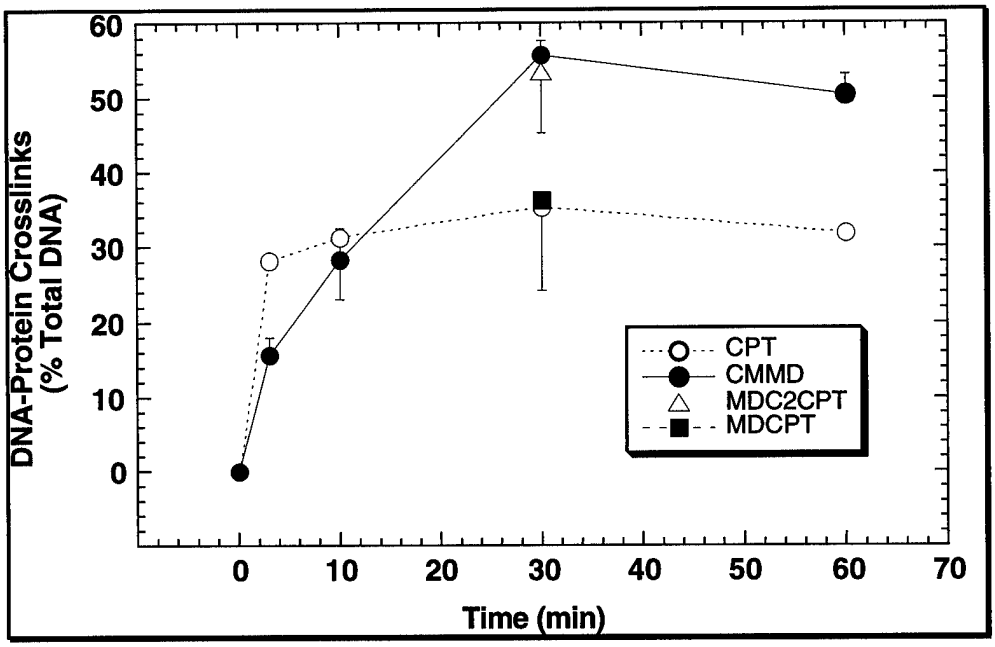

Figure 3. Time course for DNA-protein crosslinks induced in CEM nuclei by CPT analogs. CPT at 1 μM (○) is shown and compared to 0.1 μM CMMD (●), MDCPT (▲), and MDC2CPT (■). Error bars are positive for filled symbols and negative for open symbols.

C.4 Reversal of cleavable complexes formed with camptothecin analogs on plasmid DNA. We used the competitive DNA approach [27] to measuring the religation of DNA single strand breaks on plasmid DNA. An example of the data obtained for OHCPT and its analysis is given in Fig. 2B. The assumption of this method is that once topo I is dissociated from the cleavable complex, the excess of linear DNA competes for the topo I and inhibits it from re-attaching to the same or other plasmids (equations 3 and 4). Hence, the process should be a pseudo first-order dissociation of topo I from the cleavable complex, and therefore well described by a single exponential decay. This approach characterizes the stability of the complex induced by the drug, since drug dissociation is the limiting step in the process (i.e., religation in the absence of drug is rapid [32]). Other groups have used salt-induced denaturation of the DNA to produce similar decay curves [18, 20-22, 32], where the salt acts to affect the equilibria given in equation 3 by decreasing k_{12}. However, due to the unknown solubilities of the hydrophobic CPT analogs in 0.35-0.5 M NaCl, we chose to keep the ionic strength constant and use competitive DNA. We show below that the data obtained here is consistent with that obtained using the salt-induce release of cleavable complexes.

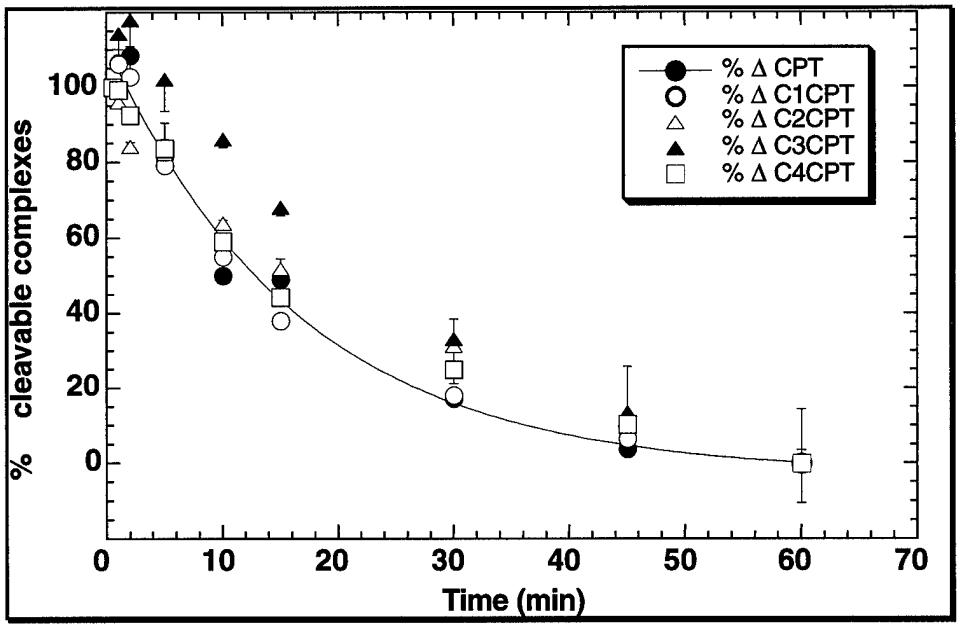

Figure 4. Kinetics of competitive DNA-induced religation of cleavable complexes formed on plasmid DNA with human topoisomerase I and (●) CPT and the 7-alkyl CPT analogs (○) C1CPT, (△) C2CPT, (▲) C3CPT, and (□) C4CPT. The solid line is for the decay in the presence of CPT. The rate constants for the disappearance of cleavable complexes with all of the compounds are given in Table 2.

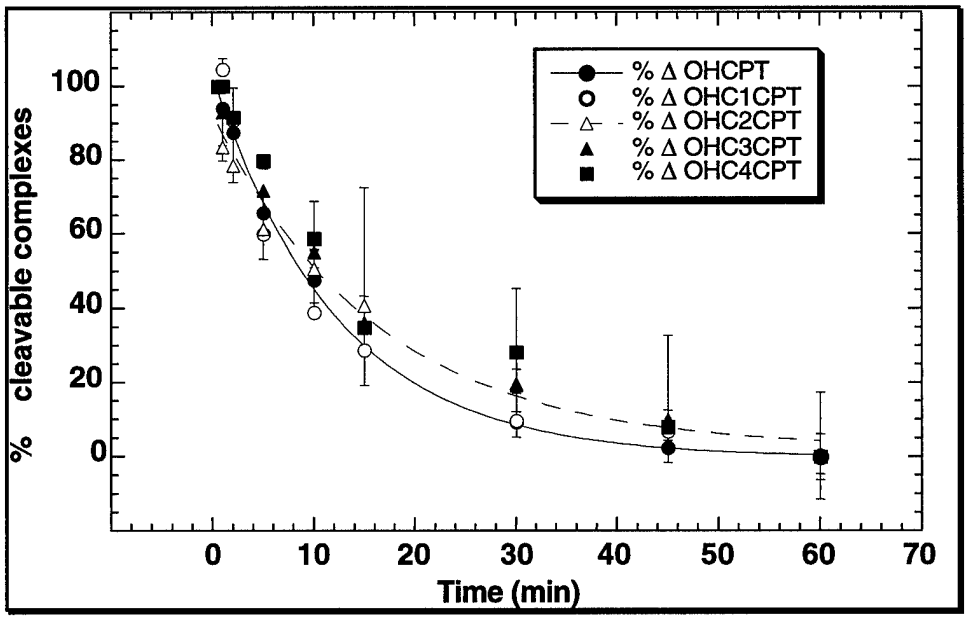

Figure 5. Kinetics of competitive DNA-induced religation of cleavable complexes formed on plasmid DNA with human topoisomerase I and the 10-hydroxy- CPT (●) OHCPT, and the 7-alkyl-10-hydroxy-CPT analogs (○) OHC1CPT, (△) OHC2CPT, (▲) OHC3CPT, and (■) OHC4CPT. The solid line is for the decay of OHCPT while the dashed line is for OHC2CPT. The rate constants for the disappearance of cleavable complexes with all of the compounds are given in Table 2. Error bars are negative for filled symbols and positive for open symbols.

There was only a minor dependence of the reversibility of the cleavable complex on the structural composition of the CPT analogs. The reversal of cleavable complexes formed with 7-alkyl CPT is shown in Fig. 4. Despite the enhanced potency in poisoning topo I and inhibiting cell growth by the 7-substituted CPT, the reversal kinetics for complexes formed by these compounds are essentially identical to those formed with CPT, and all followed a simple single exponential decay. The fitted decay curve for CPT is shown by the solid line in Fig. 4, but the τ values determined for all the analogs is given in Table 2.

When the 10-hydroxy compounds were examined, there was also only a minor, if any, dependence of kinetics of reversal on the 7-position (Fig. 5). The solid line in Fig. 5 is for OHCPT while the dashed line is shown for OHC2CPT. While the amplitudes of decay are somewhat different for the 7-alkyl OCHPT vs. OHCPT, the rate constant governing the decay is similar (Table 2). Further, despite the high potency of the OHCPT series vs. CPT in poisoning topo I and inhibiting cell growth, the rate constants governing religation by topo I in the presence of the 7-alkyl OHCPT are actually slightly larger (i.e., religation is faster) than with those of the corresponding 7-alkyl CPT analogs.

The decay of cleavable complexes stabilized with the OMeCPT was faster than that observed with CPT (Fig. 6; Table 2). Further, all of the 7-alkyl OMeCPT had identical decay curves, and hence were independent of 7-alkyl chain length. The dashed line drawn in Fig. 6 is shown for OMeC2CPT, and all the τ values are recorded in Table 2.

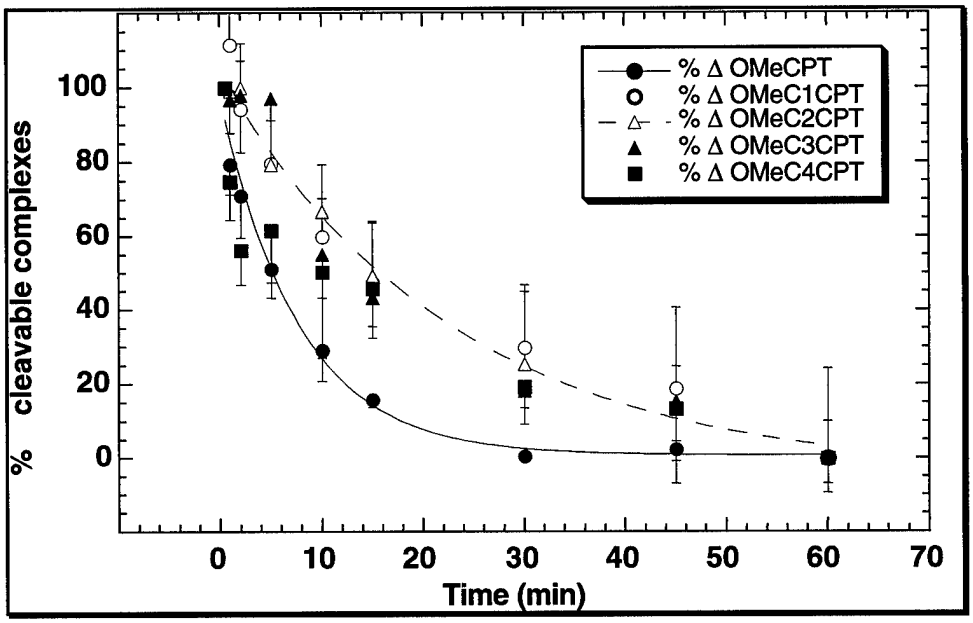

Figure 6. Kinetics of competitive DNA-induced religation of cleavable complexes formed on plasmid DNA with human topoisomerase I and the 10-methoxy CPT (●) OMeCPT and the 7-alkyl-10-methoxy CPT analogs (○) OMeC1CPT, (△) OMeC2CPT, (▲) OMeC3CPT, and (■) OMeC4CPT. The solid line is for the decay of OMeCPT while the dashed line is for OMeC2CPT. The rate constants for the disappearance of cleavable complexes with all of the compounds are given in Table 2. Error bars are negative for filled symbols and positive for open symbols.

Reversal of complex formation formed with MDCPT analogs also displayed little dependence on the 7-substitution (Fig. 7). All cleavable complexes formed with the MDCPT analogs had similar rate constants for decay (Table 2). However, all MDCPT analogs did maintain a higher number of cleavable complexes after 1 hr (~ 60%) as compared with the CPT, OHCPT, or OMeCPT series of analogs (20 -

40% of complexes remaining after 1 hr). In otherwords, the amplitude for the decay with the MDCPT analogs was lower than for the other series examined.

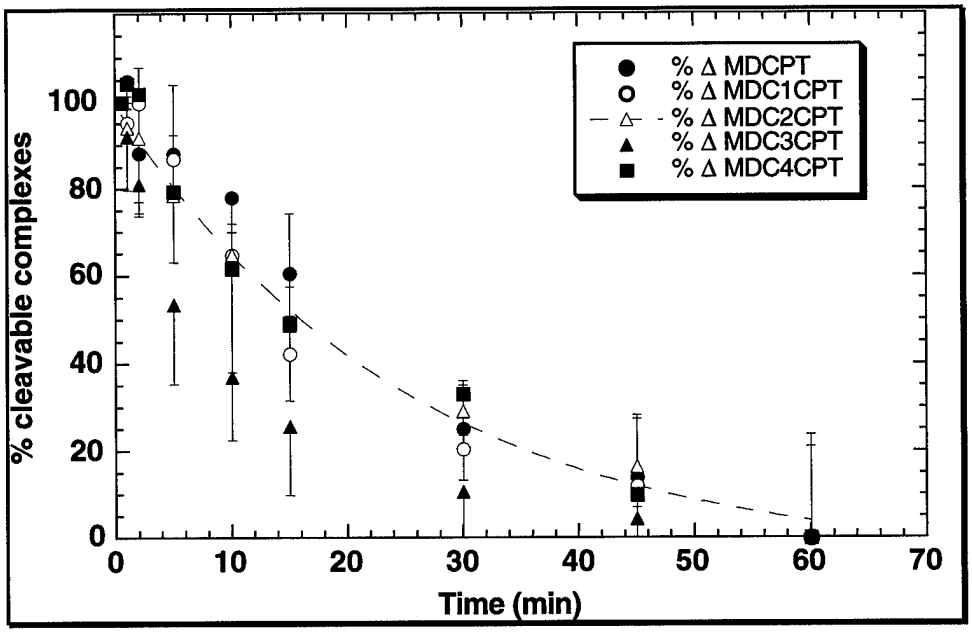

Figure 7. Kinetics of competitive DNA-induced religation of cleavable complexes formed on plasmid DNA with human topoisomerase I and the 10,11-methylenedioxy CPT analog (●) MDCPT, and the 7-alkyl-10,11-methylenedioxy-CPT analogs (○) MDC1CPT, (△) MDC2CPT, (▲) MDC3CPT, and (■) MDC4CPT. The dashed line is for MDC2CPT. The rate constants for the disappearance of cleavable complexes with all of the compounds are given in Table 2. Error bars are negative for filled symbols and positive for open symbols.

Table 2. Pseudo first-order rate constants for decay of cleavable complexes stabilized by 7,10-disubstituted camptothecins.

Camptothecin Analog	rate constant τ from plasmid[a] (min^{-1})	rate constant τ from nuclei[b] (min^{-1})
CPT	0.061 ± 0.010	0.130 ± 0.013
C1CPT	0.066 ± 0.008	
C2CPT	0.034 ± 0.006	
C3CPT	0.024 ± 0.009	
C4CPT	0.050 ± 0.005	
OHCPT	0.082 ± 0.004	
OHC1CPT	0.108 ± 0.012	
OHC2CPT	0.061 ± 0.013	

OHC3CPT	0.064 ± 0.006	
OHC4CPT	0.058 ± 0.010	
OMeCPT	0.131 ± 0.017	
OMeC1CPT	0.054 ± 0.012	
OMeC2CPT	0.044 ± 0.006	
OMeC3CPT	0.054 ± 0.014	
OMeC4CPT	0.045 ± 0.024	
MDCPT	0.028 ± 0.007	0.036 ± 0.011
C1MDCPT	0.049 ± 0.008	
C2MDCPT	0.040 ± 0.006	0.045 ± 0.019
C3MDCPT	0.114 ± 0.012	
C4MDCPT	0.045 ± 0.009	
CMMD (chloromethyl)	0.277 ± 0.048	0.088 ± 0.011

[a]Disappearance of cleavable complexes with time after addition of 100-fold excess sheared salmon sperm DNA. [b]Disappearance of protein-DNA crosslinks in CEM nuclei following suspension of nuclei in drug-free media.

C.5 Reversal of Protein-DNA Crosslinks in CEM Cell Nuclei. A comparison of reversal data from plasmid DNA vs. cell nuclei is given in Fig. 8 for selected CPT analogs. CMMD, the MDCPT analog with the potential for alkylation of DNA, shows an initially rapid, exponential reversal in plasmid DNA (Fig. 8A). However, only approximately 40% of the CMMD complexes re-ligate during this decay, as compared to almost complete reversal by CPT, OHCPT and OMeCPT (e.g., Figs. 4-6). This reduced reversal for CMMD may not be due to covalent linkage of the drug, since the data for the non-alkylating analog C2MDCPT reveal a slow, almost linear relaxation of the complexes formed by this drug—even slower than those observed for CMMD (Fig. 8A). C2MDCPT does not appear to alkylate DNA [18, 20].

Reversal of protein-DNA crosslinks in cell nuclei upon washing with drug-free media is shown in Fig. 8B. The analogs used were those in Fig. 8A. Decay curves for each analog in cell nuclei upon washing out of drug are similar to those seen with plasmid DNA (τ values recorded in Table 2). For both MDCPT and MDC2CPT, the rate constants for reversal in both plasmid DNA and cell nuclei are (within error) identical, indicating that the DNA competition method is effectively similar to drug wash out experiments. CPT and CMMD had slightly higher rate constants for complex reversal in nuclei than in plasmid DNA.

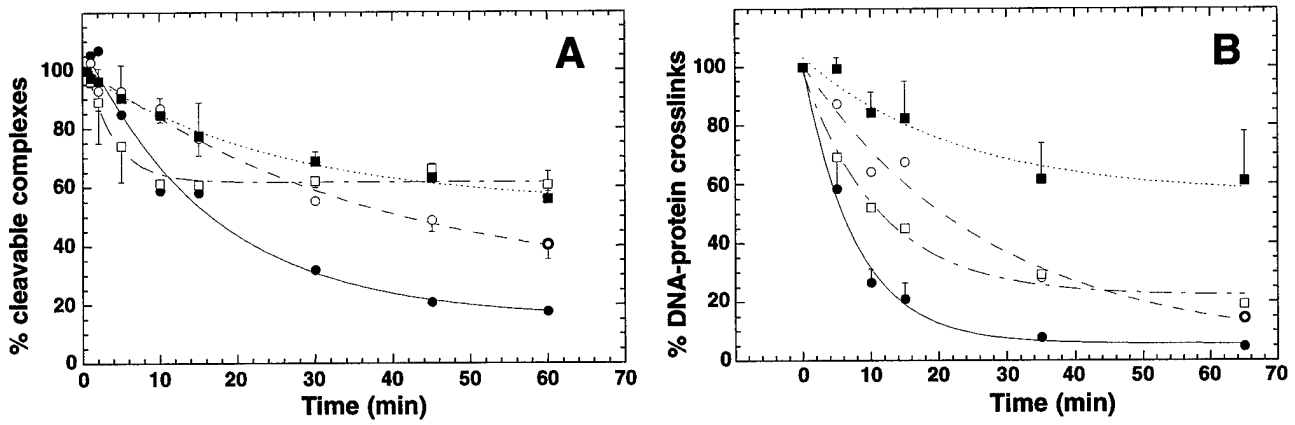

Figure 8. Comparison of cleavable complex reversal on plasmid DNA with reversal of DNA-protein crosslinks in CEM cell nuclei. Panel (A): reversal of cleavable complexes formed on pBR322 plasmids with (●) CPT, (○) MDCPT, (□) CMMD, and (■) MDC2CPT. Panel (B): reversal of DNA-protein crosslinks induced by the drugs in CEM cell nuclei (same symbols as panel A). Rate constants for reversal of DNA-protein crosslinks with the four compounds shown are given in Table 2.

In both the plasmid and nuclei, cleavable complexes are extensively reversable with CPT, however the complexes formed with MDC2CPT remained at high levels even after 1 hr in the presence of salmon sperm DNA (Fig. 8A) or after washing of the nuclei (Fig. 8B). CMMD showed similar behavior in nuclei as in plasmids, with an initial decay followed by stable complexes. However, CMMD-induced complexes reversed to a greater extent in nuclei than with plasmid DNA. As a control, the effects of formaldehyde, used as a compound forming direct covalent DPC, were completely irreversible under the same conditions (118 and 95 % of the initial DPC after 60 min with 100 and 500 μM formaldehyde, respectively; data not shown).

D. DISCUSSION OF PART I:

The evaluation of the CPT analogs reported here has been performed to advance the development of of "3rd generation" CPT analogs, with topo I poisoning activities higher than TPT, OHC2CPT (SN-38), or MDCPT. Recent reports from the Pommier laboratory [18, 21] have suggested that CPT analogs with greater potency than CPT or TPT in forming cleavable complexes might produce this enhanced activity by forming complexes that are slow to reverse. These persistent complexes would be more likely to be present during replication, and therefore be more toxic to cells. Thus, structural features of CPT analogs lending themselves to slowly-reversing cleavable complexes are important for generating improved CPT analogs with activities that might surpass CPT-11 and TPT much as CPT-11 and TPT surpassed the parent CPT.

In this report, we examined 7-alkyl- and 7-alkyl-10-substituted CPT analogs for their mechanism of *in vitro* topo I poisoning and their ability to inhibit tumor growth in cell culture. Our data indicate that all 7- or 7, 10-substituted CPT analogs are more potent than CPT in inducing topo I-mediated cleavable complexes with purified enzyme and plasmid DNA (Table 1). The MDCPT analogs of CPT are among the most potent compounds known for poisoning topo I. However, the stabilization of cleavable complexes is independent of the potency of the compounds in both poisoning of topo I on plasmid DNA or inhibiting cell growth (compare Tables 1 and 2). CPT and its 7-alkyl derivatives show rapid, exponential reversibility of the complexes formed (Fig. 4), despite the much higher potency of the 7-alkyl

derivates to poison topo I and to inhibit tumor cell growth. Similar results were observed for the OHCPT, OMeCPT, and MDCPT analogs of CPT: no correlation between the potency of the drug and its ability to stabilize cleavable complexes. Our work is in agreement with a recent paper on 9,10-disubstituted CPT analogs by Hecht and colleagues [32]. Using salt-induced religation and dissociation of topo I from a defined oligonucleotide, these authors also found no correlation between the potency of these analogs in poisoning topo I and their ability to stabilize cleavable complexes.

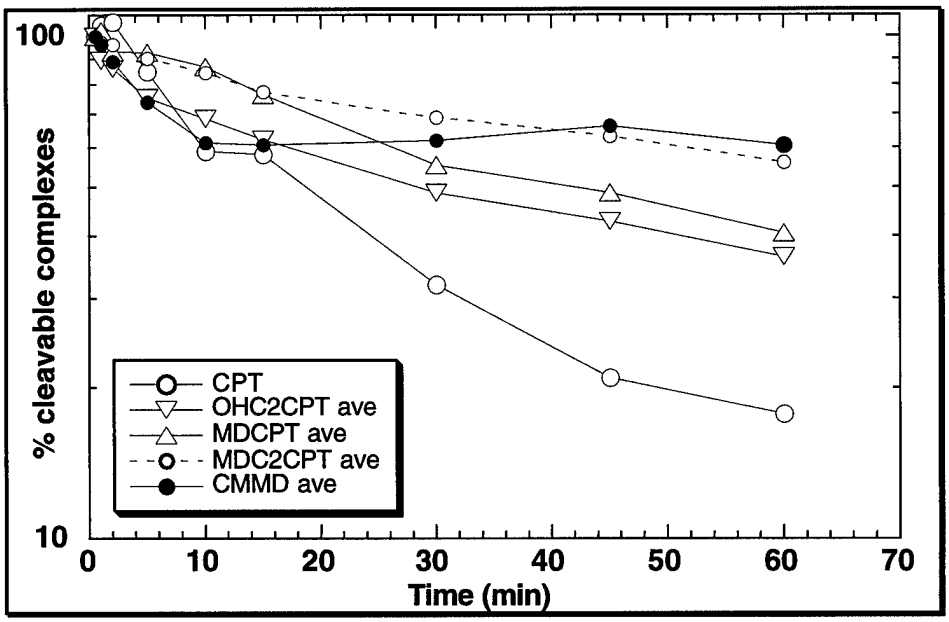

Figure 9. Persistence of cleavable complexes on plasmid DNA after addition of competitive DNA. Data are shown for (⊙) CPT, (▽) OHC2CPT, (△) MDCPT, (○) MDC2CPT, and (●) CMMD. Note that while the percentage of cleavable complexes remaining after 1 hr is markedly different, the rate constants for the complex reversal process are similar for all the analogs examined (Table 2).

Our results, and those of Hecht and colleagues, point out a critical facet of topo I-DNA poisoning that may not be readily apparent when considering "longer-lived" cleavable complexes. In Fig. 9, we have re-cast data from Figs. 4-7 on a semi-log plot using the CPT analogs and symbols taken from Fig 3B of [18]. Comparison of our data, using competitive DNA, and their data, using salt-induced topo I dissociation, indicates that the data are remarkably similar with regard to the "stability" of the cleavable complexes. That is, in both of our studies, after a 1 hr period, more complexes exist when MDC2CPT and CMMD are used, and these in turn are greater in number than MDCPT- and OHC2CPT- induced complexes, with CPT-induced complexes even lower. However, note from Table 2 that the rate constants τ for all of these compounds are similar, and the τ value for OHC2CPT is identical to that for CPT. Hence, the "stability" of the complexes, as defined by the rate constants, are <u>not</u> correlated with the potency of the analogs in poisoning topo I or inhibiting cell growth.

Both the competitive DNA method used here and the salt-induced religation methods used elsewhere are examples of chemical relaxation experiments, where a steady-state is rapidly perturbed by addition of some component, and the system under study relaxes to a new steady-state (these methods are exhaustively reviewed in [33]). The concentration difference in components between the new steady-state and the old gives rise to the amplitude of the perturbation (i.e, A in eq. 3). The decay time is given by τ. Hence the highly potent CPT analogs may have a smaller A, since they poison topo I at very low concentrations, but generate cleavable complexes with identical "stabilities". In both the pBR322 cleavage assays and the cell growth assays, we are examining steady-state situations (i.e., constant amounts of drug present). Hence, it is not surprising that, in these assays, the potency of the drug in inhibiting cell growth more closely relates to the potency in poisoning topo I on plasmid DNA. In both situations, there

is little or no dependence on kinetic parameters of the drug-DNA-topo I ternary complex. It should be noted, however, that while the EC_{50} values for topo I poisoning are more reflective of the *in vitro* growth inhibition IC_{50} values by the CPT analogs (that is compounds with lower EC_{50} values also had lower IC_{50} values), the actual correlation coefficient for these two parameters is low ($r = 0.5$ for BT-20 cells) or non-existent ($r = 0.1$ for MDA-231 cells). This is most likely due to differences in accumulation of the analogs within cells.

In contrast to the usual topo I poisoning assays and cell growth inibition assays, clinical use of CPT analogs does involve a kinetic component. During a typical administration of CPT-11 or TPT, the blood concentration of SN-38 or TPT will rise then fall with time. Since cell kill in S-phase is also time dependent (i.e., it requires collision of the replication fork with topo I-DNA complexes), clearly CPT analogs that form slow decaying ternary complexes might be desirable to maximize cancer cell killing. As suggested recently [18, 21, 22], development of a new generation of CPT analogs with antitumor activities greater than CPT-11 or TPT may include drugs designed to stabilize cleavable complexes to a much greater extent than the 7- and 10-substituted analogs examined here. Ideally, compounds such as the CMMD analog, which can alkylate DNA, may be most useful in forming stabile, irreversible cleavable complexes. However, only a small fraction of the available CMMD has been shown to covalently bind to DNA [18, 21] and therefore these complexes rapidly reverse (Fig. 8). Hence new, more reactive alkylating CPT analogs may prove more effective in their cytotoxic activity. We anticipate that the data presented here should aid in development of such compounds.

E. ACKNOWLEDGEMENTS FOR PART I:

Bogdan Vladu, Jan M. Woynarowski, Govindarajan Manikumar, Mansukhlal C. Wani, Monroe E. Wall, and Randy M. Wadkins contributed to the work described above. We also thank Dr. Shih-Fong Chen for initiating aspects of this work. We thank Alex V. Trevino and William G. Chapman for excellent technical assistance with the DPC determinations.

F. INTRODUCTION TO PART II: "Water Soluble 20(S)-Glycinate Esters of 10,11-Methylenedioxycamptothecins are Highly Active Against Human Breast Cancer Xenografts."

F.1 Background. In 1966, Wall and colleagues discovered that camptothecin (CPT) was the component in the extract from the stem of the Chinese tree *Camptotheca acuminata* [1] active against L1210 murine leukemia cells. Early clinical trials with the water soluble, E-ring open lactone sodium salt of CPT afforded results that were inconclusive [2-4]. It was soon discovered that this form of CPT was an inactive one, and clinical trials were discontinued.

Further development of topoisomerase I (topo I) inhibitors for cancer therapy was stimulated by the characterization of CPT as a specific topo I inhibitor [5, 6]. Topo I relaxes DNA supercoiling by making transient single-strand breaks [7, 8]. These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex [5, 6]. CPT and analogs specifically and reversibly stabilize cleavable complexes by inhibiting their religation (reviewed in [10]). The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes [6, 11, 12].

F.2 Water-soluble CPT analogs. CPT-11 (7-ethyl-10-[4-[1-piperidino]-1-piperidino]carbonyloxy CPT; Irinotecan; Camptosar) was developed as a water-soluble CPT analog [25]. It has recently received regulatory approval in the United States and elsewhere for patients with metastatic colorectal carcinoma who previously received 5-fluorouracil. This analog of 10-hydroxy CPT possesses an ethyl group at the 7-position of CPT and a piperidino-1-piperidino carbamate ester at the 10-position. The latter moiety

conveys water solubility and makes CPT-11 a prodrug that undergoes de-esterification by carboxylesterases *in vivo* to yield SN-38 (7-ethyl-10-hydroxy CPT).

In clinical use of CPT-11 in North America, neutropenia and diarrhea are the most common toxicities encountered, with diarrhea occurring in two forms. The most common form of diarrhea, a delayed-onset diarrhea, occurs usually after the second or third dose of CPT-11. The other form is a dose-related toxicity occurring during peritreatment period. This latter, acute form of diarrhea is usually associated with abdominal cramping, vomiting, flushing, visual (accommodation) disturbances, lacrimation, salivation, bradycardia, and diaphoresis [34, 35]. These effects appear to be due to the cholinergic actions of CPT-11, which is an inhibitor of acetylcholinesterase *in vitro*. The inhibition of acetylcholinesterase has recently been traced to the 10-(piperidino-1-piperidino) moiety of CPT-11 [36]. The extreme effects of diarrhea can be mitigated both by the dosage control and regime, and aggressive use of loperamide or atropine [37, 38].

Upon intravenous infusion, CPT-11 plasma concentrations are maximal immediately following the end of the infusion, whereas SN-38 plasma concentrations peak approximately 2 hours later. Significant interpatient variability in plasma concentrations and AUC are observed with CPT-11 [39-43]. The variability in the kinetics of SN-38 formation, as well as in peak SN-38 concentrations, suggest that variations in carboxylesterase converting enzymes may, in part, account for these effects, and, consequently, this variability may be a critical determinant of toxicity and response.

F.3. Novel, highly-potent Water-soluble CPT analogs. A number of other 7- and 10-substituted and 10,11-disubstituted camptothecins have been developed [15-20]. These include the highly potent 10,11-methylenedioxycamptothecin (MDCPT) and 7-chloromethyl-10,11-methylenedioxycamptothecin (CMMD) [18, 20], whose structures are given in Figure 10. The latter compound is of particular interest as it is capable of forming a covalent complex with DNA through nucleophilic displacement of the chlorine moiety by DNA while in the cleavable complex. The development of water-soluble analogs of 10,11-methylenedioxy camptothecins was performed, with the specific intent to develop compounds that improve water solubility without sacrificing the stability of the E-ring ketone (critical for biological activity), and which do not require enzymatic conversion to the active compound.

Analogs of MDCPT and CMMD have been developed that would appear to satisfy this search. Substitution at the 20(S)-position of CPT with a glycinate ester has recently been reported [16]. The 20(S)-glycinates are more water soluble than their parent compounds. The glycinates are converted by aqueous hydrolysis to their active 20(S)-hydroxy forms on a time scale comparable to *in vivo* conversion of CPT-11 to SN-38 by esterases. In this report, we describe the *in vivo* use of the glycinate esters of the highly potent MDCPT and CMMD analogs for treatment of human breast cancer xenografts, and demonstrate these analogs are as active as CPT-11 but may avoid some problems observed clinically with CPT-11.

G. MATERIALS AND METHODS:

G.1 Camptothecin Analogs. The synthesis of MDCPT, MDCPT•Gly, CMMD, and CMMD•Gly (Fig. 10) used in these studies has been described elsewhere [16, 19]. CPT-11 and SN-38 for *in vitro* studies was synthesized according to [25]. CPT-11 for the *in vivo* studies was obtained from the Cancer Therapy and Research Center pharmacy as prepared by Pharmacia-Upjohn Pharmaceuticals.

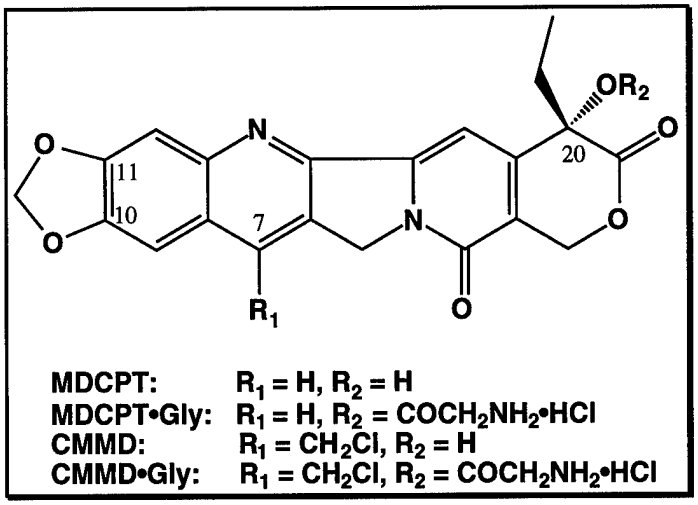

Figure 10. Structure of camptothecin analogs. 10,11-methylenedioxycamptothecin (MDCPT), 7-chloromethyl-10,11-methylenedioxycamptothecin (CMMD), and their corresponding 20(S)-glycinate esters (MDCPT•Gly and CMMD•Gly).

G.2. Aqueous Solubility and Stability of Camptothecin Analogs. The enhanced solubility of the glycinate esters or CPT-11 analogs *vs.* their un-esterified parent compounds was determined using octanol-water partitioning. A ~10 μM solution of each analog was prepared by diluting a DMSO stock solution of each into 2 mL of 1-octanol. The fluorescence intensity from the octanol solution was measured, and the solution subsequently vigorously extracted for 2 min with an equal volume of 0.1 M sodium acetate, pH 5.0. Following extraction, the fluorescence from the octanol solution was again measured, and the octanol-buffer partition coefficient *P* determined from the ratio of the initial to final octanol solution fluorescence.

The kinetics of conversion of MDCPT•Gly and CMMD•Gly to their parent MDCPT and CMMD compounds was measured by HPLC. MDCPT•Gly or CMMD•Gly were dissolved in phosphate buffered saline (PBS), pH 7.5, at 1 mg/mL. The solutions were incubated at 37.5 °C with stirring. Aliquots of the solution were removed at 0, 0.5, 1, 3, 6, and 24 hr. Samples were extracted with $CHCl_3$:methanol (4:1 v/v), and the organic layer dried in vacuo. The resulting dry extract was redissolved in methanol and run on a C18 reverse phase HPLC column, with 3:2 methanol:water (v/v) as the mobile phase. Elution of MDCPT•Gly and MDCPT were monitored by absorbance at 320 nm.

The remaining aqueous layer of the aliquot was acidified with HCl, and the procedure repeated to account for the total amount of MDCPT and CMMD present. The half-lives for MDCPT•Gly and CMMD•Gly conversion to the parent compounds under these conditions were determined to be 6 hr and 1.5 hr, respectively.

The kinetics of *in vivo* hydrolysis of MDCPT•Gly and CMMD•Gly glycinate esters were monitored by injecting B6D2F1 mice ip with the MTD of MDCPT•Gly or CMMD•Gly (10 or 15 mg/kg, respectively). At 0.25, 0.5, 1, 2, 4, and 24 hr, blood samples were taken from 3 mice, combined, and the plasma isolated. Plasma samples were snap-frozen with liquid nitrogen until processed.

A 50 μL aliquot of the plasma samples was mixed with 300 μL of 1 N HCl in methanol to precipitate plasma proteins. The samples were briefly centrifuged to remove precipitated protein. The clarified methanol solutions were analyzed by HPLC using a C8 column with 10 mM potassium phosphate buffer (pH 2.5):methanol (52:48) as the mobile phase. Detection of products was via fluorescence using an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Under these conditions, the glycinate esters were present in four forms: protonated glycinate ester of the lactone, protonated glycinate of the hydroxy acid, and non-protonated glycinate esters of the lactone and hydroxy acid. These four hydrophilic forms of MDCPT•Gly and CMMD•Gly eluted as broad peaks at 4-10 min or

2-8 min, respectively, after injection. These broad peaks were integrated together and are considered total glycinate. The plasma total glycinate concentrations of both compounds peaked after 0.5 hr, and then decayed as a single exponential with time. The *in vivo* half lives for the MDCPT•Gly and CMMD•Gly were 0.5 and 1.3 hr, respectively. This conversion rate is only slightly faster than that described above for buffer.

Under our HPLC conditions, the released active form of the drugs eluted as a single peak at 12-14 min and 10-11 min for MDCPT and CMMD, respectively. *In vivo*, only a small amount of free CMMD could be detected relative to the CMMD•Gly. We interpret this to mean that, since CMMD is a reactive compound, it covalently binds with serum proteins or other macromolecules and cannot be efficiently extracted from plasma. MDCPT, however, could be easily detected. Plasma levels of MDCPT peaked at 2 hr following ip injection. Hence disappearance of the glycinates corresponded with appearance of the active form of the drug.

G.3. Acetylcholinesterase Inhibition Assays. Human acetylcholinesterase was obtained from Sigma. Inhibition of the enzyme by the CPT analogs was determined using 3 mM o-nitrophenyl acetate, as previously described [44, 45]. IC_{50} values were determined using at least 6 concentrations of CPT analog. Inhibition measurements were made within 10 min of addition of glycinate esters such that only trivial amounts of the non-glycinate parents were present in solution.

G.4. *In vivo* activity of CMMD•Gly and MDCPT•Gly. Female nude mice weighing approximately 20 g were implanted subcutaneously by trocar with fragments of either MDA-231 or MX-1 human breast carcinomas harvested from subcutaneously growing tumors in nude mice hosts. When tumors were approximately 5 mm x 5 mm in size (10 days after inoculation for MDA-231 and 12 days after inoculation for MX-1), the animals were pair-matched into treatment and control groups. Each group contained 8 tumored mice, each of which was ear-tagged and followed individually throughout the experiment. The administration of drugs or vehicle began the day the animals are pair-matched (Day 1) and all injections were done i.p. The glycinate esters were formulated for injection in 0.25 % methylcellulose, 2 % Tween 80. For MDA-231, MDCPT•Gly was administered at 1.0 and 0.5 mg/kg on a qdx5 schedule and at 10.0 and 5.0 mg/kg on a qdx1 schedule. CMMD•Gly was administered at 7.5 and 3.75 mg/kg on a qdx5 schedule and at 15.0 and 7.5 mg/kg on a qdx1 schedule. These concentrations were the MTD and half-MTD for each compounds. CPT-11 was used as a positive control and was given at 100 mg/kg on a weekly x 3 schedule. For MX-1, MDCPT•Gly was administered at 0.5 and 0.25 mg/kg on a qdx5 schedule and at 5.0 and 2.5 mg/kg on a qdx1 schedule. CMMD•Gly was administered at 7.5 and 3.75 mg/kg on a qdx5 schedule and at 15.0 and 7.5 mg/kg on a qdx1 schedule. CPT-11 was used as a positive control and was given at 100 mg/kg on a weekly x 3 schedule.

Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on Day 1. These tumor measurements were converted to mg tumor weight by L^2 x $W/2$ (where L is length and W is width), and from these calculated tumor weights, the termination date was determined. The experiment was terminated when control tumors reached a size of 2000 mg. Upon termination, all mice were weighed, sacrificed, and their tumors excised. Tumors were weighed, and the mean tumor weight per group was calculated. In this model, the mean treated tumor weight / mean control tumor weight x 100% (T/C) was subtracted from 100% to give the tumor growth inhibition (TGI) for each group.

With these agents, the final weight of a given tumor was subtracted from its own weight at the start of treatment on Day 1. This difference divided by the initial tumor weight is the % shrinkage. A mean % tumor shrinkage was calculated from data from the mice in a group that experienced regressions

H. RESULTS FOR PART II:

H.1 Aqueous solubility of CPT analogs. The octanol:buffer partition coefficient *P* for the 6 CPT analogs are given in Table 3. The piperidino-1-piperidino carbamate ester of CPT-11 doubles the aqueous solubility of this compound relative to SN-38. Both MDCPT and CMMD are substantially less soluble than SN-38 in an aqueous solution. However, addition of the 20(S)-glycinate in MDCPT•Gly and CMMD•Gly increase the solublity of these compounds 16- and 4-fold, respectively. The glycinates show similar solubility to SN-38 but are slightly less soluble than CPT-11.

Table 3. Octanol/Buffer partition coefficient for camptothecin analogs.

Camptothecin analog	*P*
MDCPT	16.2 ± 0.7
MDCPT•Gly	0.99 ± 0.03
CMMD	6.3 ± 0.9
CMMD•Gly	1.6 ± 0.3
SN-38	0.89 ± 0.01
CPT-11	0.52 ± 0.03

***P* is ratio of molar concentration of analog in 1-octanol relative to 0.1 M sodium acetate, pH 5.0.**

H.2. Inhibition of Acetylcholinesterase. As shown in Table 4, CPT-11 is a potent inhibitor of human acetylcholinesterase (AcChE). This is presumably due to the piperidino-1-piperidino carbamate group of CPT-11, as SN-38 is 37-fold less effective as an inhibitor of AcChE. In contrast to CPT-11, both MDCPT•Gly are CMMD•Gly much less (20- and 38-fold) potent as inhibitors of AcChE.

Table 4. Inhibition of human acetylcholinesterase by camptothecin analogs.

Camptothecin analog	IC50[†] (μM)
MDCPT•Gly	19.5 ± 6.5
CMMD•Gly	10.3 ± 2.5
SN-38	19.4 ± 10.3
CPT-11	0.52 ± 0.07

[†]Concentration of analog needed to inhibit human acetylcholinesterase activity by 50%, at 3 mM o-nitrophenyl acetate.

H.3. In vivo studies of the antitumor activity of water-soluble forms of MDCPT and CMMD. The results of the glycinate compounds against MX-1 human breast tumor and MDA-231 human breast tumor are given in Figures 11 and 12, respectively, and are compared to CPT-11.

MDCPT•Gly and CMMD•Gly both showed considerable antitumor activity against the MX-1 human breast tumor xenograft model (Figure 11). MDCPT•Gly, at 0.25 and 0.5 mg/kg on a qd x 5 schedule, resulted in 3/8 and 8/8 complete responses, respectively. MDCPT•Gly at 5 mg/kg on a qd x 1 schedule produced 7 complete responses while one complete response was noted at 2.5 mg/kg using the same dosing schema. MDCPT•Gly was, in general, well tolerated with one toxic death at 5 mg/kg on a qd x 1 schedule.

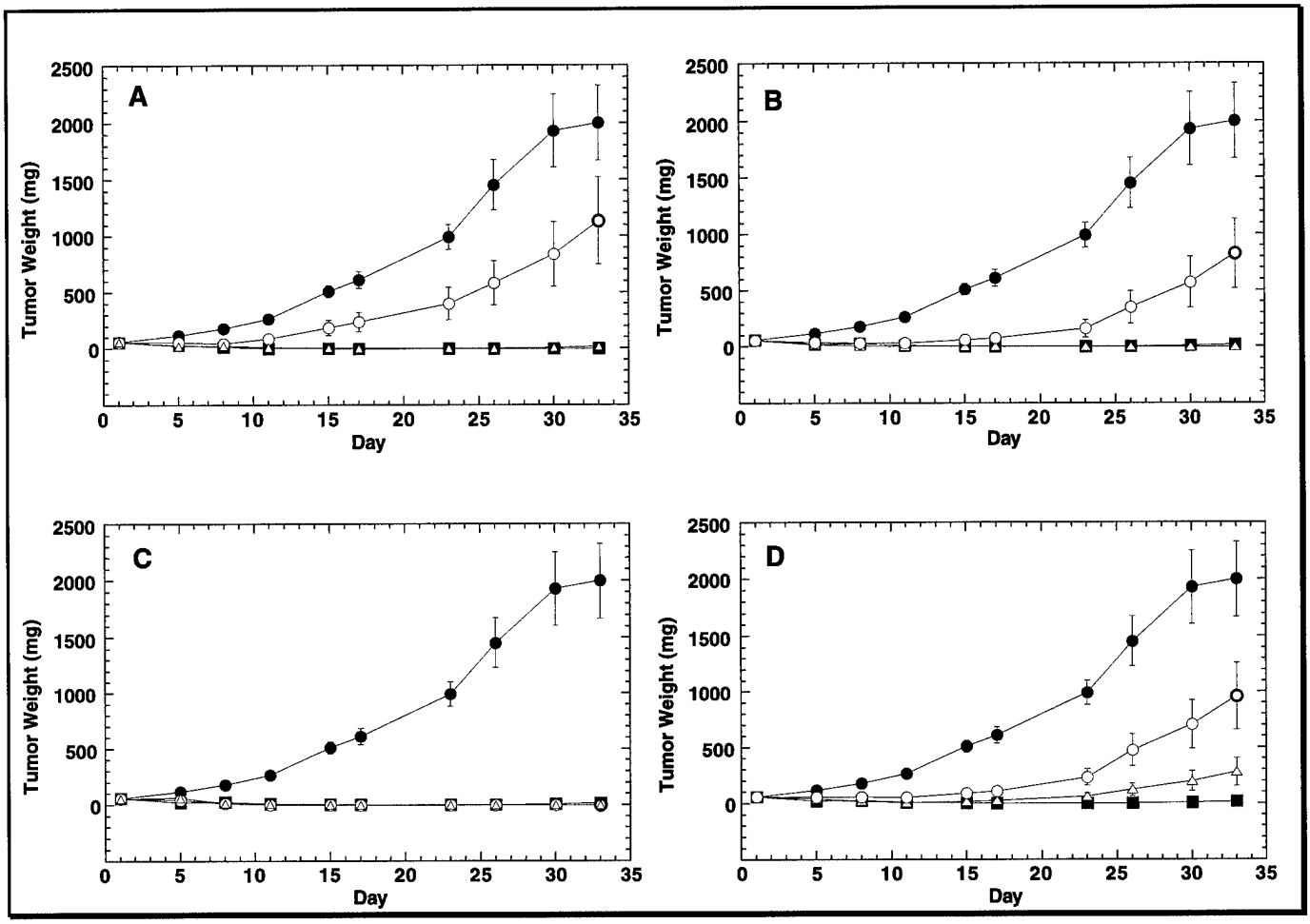

Figure 11. Activity of MDCPT•Gly, CMMD•Gly, and CPT-11 against MX-1 tumor xenografts. Tumor weights in nude mice are shown for (●) control and (■) CPT-11 (100 mg/kg/week) in all panels. (A) Tumor weights when dosing MDCPT•Gly at (○) 0.25 mg/kg, qdx5 and (△) 0.5 mg/kg, qdx5. (B) Tumor weights when dosing MDCPT•Gly at (○) 2.5 mg/kg, qdx1; and (△) 5 mg/kg, qdx1. (C) Tumor weights when dosing CMMD•Gly at (△) 3.8 mg/kg, qdx5; (○) 7.5 mg/kg, qdx5. (D) Tumor weights when dosing CMMD•Gly at (○) 7.5 mg/kg, qdx1; and, (△) 15 mg/kg, qdx1.

CMMD•Gly, at 3.75 and 7.5 mg/kg on a qd x 5 schedule, resulted in complete responses in all animals bearing MX-1 tumors. When this agent was administered on the qd x 1 schedule, 3 and 1 complete responses were seen at 7.5 and 15 mg/kg, respectively. The mean final tumor weight was 868.4 mg at 7.5 mg/kg and was 177.5 mg at 15 mg/kg (controls-1227.4 mg) using the qd x 1 schedule. CMMD•Gly was well tolerated with no toxic deaths or substantial change in body weight. CPT-11 (100 mg/kg) was used as a positive control and produced 7/8 complete responses using a weekly x 3 schedule.

Against MDA-231 (Figure 12), MDCPT•Gly at 1 mg/kg on a qd x 5 schedule was too toxic (8/8 toxic deaths) to evaluate the efficacy of this agent. Using this schedule at 0.5 mg/kg, no antitumor activity

was observed. A mean final tumor weight of 2414.5 mg compared to 1959.5 mg in vehicle controls and 507.3 mg in animals treated with CPT-11 resulted. MDCPT•Gly at 10 mg/kg on a qd x 1 schedule was, again, too toxic to evaluate antitumor activity of this agent. When this agent was administered at 5 mg/kg on the qd x 1 schedule, the final tumor weight (673.0 mg; TGI = 68.0) was significantly ($p < 0.05$) decreased compared to controls and was comparable to animals treated with CPT-11 (507.3 mg; TGI = 73.0). There were no partial or complete responses observed with administration of MDCPT•Gly using this tumor model.

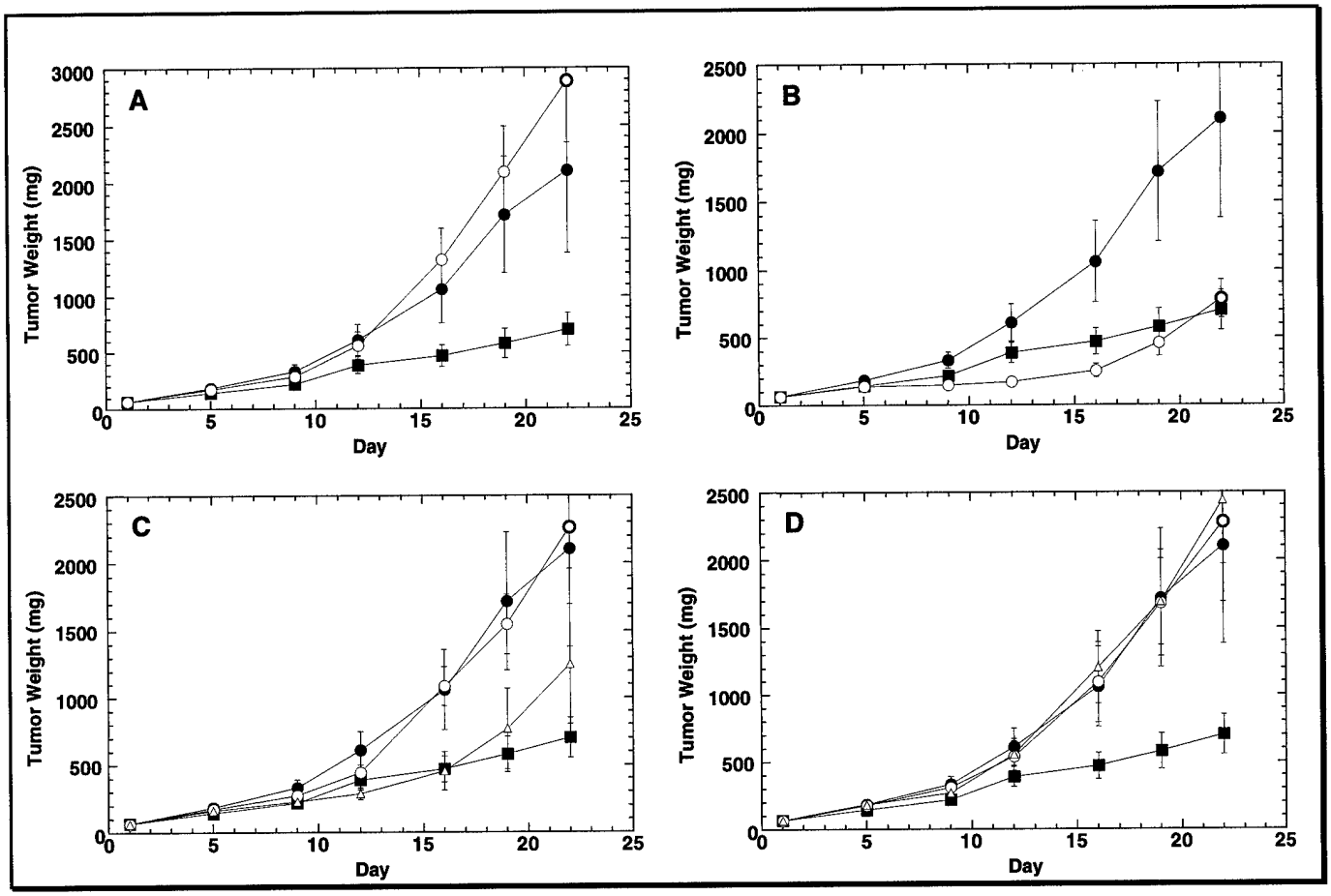

Figure 12. Activity of MDCPT•Gly, CMMD•Gly, and CPT-11 against MDA-231 tumor xenografts. Tumor weights in nude mice are shown for (●) control and (■) CPT-11 (100 mg/kg/week) in both panels. (A) Tumor weights when dosing MDCPT•Gly at (○) 0.5 mg/kg, qdx5. (B) Tumor weights when dosing MDCPT•Gly at (○) 5 mg/kg, qdx1. (C) Tumor weights when dosing CMMD•Gly at (○) 3.8 mg/kg, qdx5 and (△) 7.5 mg/kg, qdx5. (D) Tumor weights when dosing CMMD•Gly at (○) 7.5 mg/kg, qdx1; and, (△) 15 mg/kg, qdx1.

CMMD•Gly at 3.75 mg/kg on a qd x 5 schedule was inactive against the MDA-231 tumor model. When this agent was administered at 7.5 mg/kg on the same schedule, modest antitumor activity was observed with a final tumor weight of 1080.5 and TGI of 46.5. CMMD•Gly at 7.5 and 15 mg/kg on a qd x 1 schedule was inactive against this tumor model. No partial or complete responses were noted with CMMD•Gly and the MDA-231 tumor model. This agent was well tolerated with no toxic deaths or substantial change in body weight.

Administration of CPT-11 resulted in a TGI of 73.3 with one partial response of 53%. The final tumor weight following CPT-11 (507.3-CPT-11 versus 1959.5-controls) administration was significantly less than vehicle treated controls.

I. DISCUSSION FOR PART II:

In this part, we examine the use of 20(S)-glycinate esters of highly potent 10,11-methylenedioxy analogs of camptothecin, MDCPT and CMMD. The parent MDCPT and CMMD are as active or significantly more active than SN-38 against three breast cancer cell lines. However, MDCPT and CMMD are substantially less water soluble than SN-38 or CPT-11, making them difficult to use in animal studies. In contrast, the glycinate esters of these compounds are significantly more water soluble, and have been used for *in vivo* studies against MX-1 and MDA-231 breast cancer models. Importantly, the glycinate esters are not inhibitors of human acetylcholinesterase, nor do they require enzymatic conversion to the parent, active analogs.

The glycinate esters of MDCPT and CMMD were examined *in vivo* by comparing MDCPT•Gly, CMMD•Gly, and CPT-11 against MX-1 and MDA-231 human tumor xenografts (Figs. 2 and 3). In these studies, MDCPT•Gly and CMMD•Gly showed similar activity against both tumor models, and were either equivalent to CPT-11 (in the MX-1 model; Fig. 11) or only slightly less active than CPT-11 (in the MDA-231 model; Fig. 12). While CMMD is capable of topo I-mediated alkylation of DNA [18, 20], this did not result in enhanced activity of CMMD against tumor xenografts in the animal models using our dosing schedule.

A number of attempts have been made to modify the 20(S)-OH group of CPT, either for improved water-solubility or to prevent opening of the E-ring lactone. These include replacing the 20(S)-OH group with hydrogen or fluorine, or derivatizing the 20(S)-OH group with acetylation [16, 17, 46, 47]. None of these modifications have been successful, as all of these changes have resulted in inactive, less cytotoxic compounds. Our results suggest that 20(S)-glycinate esters of camptothecin and its more potent analogs can be synthesized and used clinically when water-solubility is needed in an analog. The 20(S)-glycinate analogs enhance water solubility without sacrificing the activity of the compounds. Further, the poor inhibition of acetylcholinesterase by the glycinate esters as compared to CPT-11 suggests that the cholinergic phenomena associated with CPT-11 may be avoided by using glycinate esters as solubilizing moieties.

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K. SUMMARY OF PROGRESS MADE DURING FUNDING OF DAMD17-96-1-6008

As a direct result of funding this grant proposal, we have identified a candidate compound, MDCPT•Gly, that has numerous characteristics that make it a good choice for clinical trials as a drug for treatment of patients with breast cancer. MDCPT•Gly does not require enzymatic conversion as CPT-11 does, and therefore (i) is not dependent on individual expression of carboxyesterases, and (ii) is not an inhibitor of acetylcholinesterase (consequently having reduce toxic side-effects than CPT-11). The compound is as active as CPT-11 against two breast cancer xenografts (MX-1 and MDA-231) in mice, and hence retains the high potency of its conversion product MDCPT. However, the glycinate ester moiety makes MDCPT•Gly almost as soluble in water as CPT-11.

The interesting results from our studies in year 1 and 2 of this grant have allowed us to obtain a National Institute of Health grant to continue developing CPT analogs with longer-lived cleavable complexes. Based on these data, we are also presently pursuing funding to take MDCPT•Gly into clinical trials as a potential new agent for the treatment of patients with breast cancer.

L. PUBLICATIONS, PRESENTATIONS, AND PERSONNEL SUPPORTED BY DAMD17-96-1-6008.

L.1. PUBLICATIONS:

Vladu, B., Woynarowski, J. M., Manikumar, G., Wani, M.C., Wall, M.E., Von Hoff, D.D., and Wadkins, R.M., "7- and 10-Substituted Camptothecins: Dependence of Topoisomerase I-DNA Cleavable Complex Formation and Stability on the 7- and 10-Substituents", submitted to *Molecular Pharmacology.*

Wadkins, R.M., Potter, P.M., Vladu, B., Marty, J., Mangold, G., Weitman, S., Manikumar, G., Wani, M.C., Wall, M.E., and Von Hoff, D.D. (1999) "Water Soluble 20(S)-Glycinate Esters of 10,11-Methylenedioxy Camptothecins are Highly Active Against Human Breast Cancer Xenografts", *Cancer Research.*(in press).

L.2. PRESENTATIONS:

Vladu, B., Manikumar, G., Wall, M. E., Wani, M. C., Von Hoff, D. D., and Wadkins, R. M., "7-Alkyl-10-substituted Camptothecin Analogs: Dependence of Biological Activity and Topo I Inhibition on Length of the 7-Alkyl Group.", 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, PA, Apr. 10-14, 1999.
Proc. Amer. Assoc. Cancer Res. (1999) **40**, 110.

Baker, S.D., Wall, M.E., Wani, M.C., Wadkins, R.M., "Correlation of Structural Composition of Camptothecin (CPT) Analogs and their Ability to Affect Subcellular Distribution of Topoisomerase I (topo I).", 89th Annual Meeting of the American Association for Cancer Research, New Orleans, LA, Mar. 28-Apr. 1, 1998.
Proc. Amer. Assoc. Cancer Res. (1998) **39**, 421.

Wadkins, R.M., Chen, S.-F., Dexter, D.L., Wall, M.E., Wani, M.C., and Von Hoff, D.D., "DNA Topoisomerase I-Targted Therapy for Breast Cancer.", Dept. of Defense Breast Cancer Research Program Meeting, *Era of Hope*, Oct. 31-Nov. 4, 1997; Proceedings vol. 3, p. 819.

L.3. PERSONNEL RECEIVING PAY FROM THIS GRANT:

Daniel D. Von Hoff, Randy M. Wadkins, Bogdan Vladu, Jennifer Marty, Gina Mangold, Steve Weitman, Govindarajan Manikumar, Mansukh C. Wani, Monroe E. Wall and Shih-Fong Chen.

M. Progress toward Statement of Work

According to our original statement of work , the following progress has been made. Note that year 4 of the grant, primarily concerning nititdine analogs, was deleted by the reviewers.

Goal	Research Triangle Institute	Institute for Drug Development
1	Completed	Completed.
2	Completed	Completed
3	Completed	Completed (only Glycinate compounds were examined)
4	Completed (Pyrrazalo compound synthesized but found inactive)	Completed. Goal changed to examining cleavable complex stability with biological activity.
5	Completed (B-1 and B-2 synthesized. B-4 in preparation)	Completed (Pyrrazalo compound inactive)
6	Completed. No active compounds identified	Partially completed. Early indication (not shown) is that B-2 does not covalently label DNA.
7	Goal deleted by reviewers	Completed. No active compounds identified
8	These compounds were deemed (due to the data presented earlier) to be unlikely to be very active. Hence this goal was completed early by termination.	Goal deleted by reviewers
9	Partially completed. Extensive work has been made toward this goal, but compound C-10 has not been completely synthesized.	Completed. MDCPT•Gly selected for likely clinical trial. Scale-up of this compound would be done at that time.

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